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Severity of Acute Infectious Mononucleosis Correlates with Cross-Reactive Influenza CD8 T-Cell Receptor Repertoires

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ABSTRACT Fifty years after the discovery of Epstein-Barr virus (EBV), it remains unclear how primary infection with this virus leads to massive CD8 T-cell expansion and acute infectious mononucleosis (AIM) in young adults. AIM can vary greatly in severity, from a mild transient influenza-like illness to a prolonged severe syndrome. We questioned whether expansion of a unique HLA-A2.01-restricted, cross-reactive CD8 T-cell response between influenza virus A-M1₅₈ (IAV-M1) and EBV BMLF1₂₈₀ (EBV-BM) could modulate the immune response to EBV and play a role in determining the severity of AIM in 32 college students. Only *ex vivo* total IAV-M1 and IAV-M1+EBV-BM cross-reactive tetramer⁺ frequencies directly correlated with AIM severity and were predictive of severe disease. Expansion of specific cross-reactive memory IAV-M1 T-cell receptor (TCR) V β repertoires correlated with levels of disease severity. There were unique profiles of qualitatively different functional responses in the cross-reactive and EBV-specific CD8 T-cell responses in each of the three groups studied, severe-AIM patients, mild-AIM patients, and seropositive persistently EBV-infected healthy donors, that may result from differences in TCR repertoire use. IAV-M1 tetramer⁺ cells were functionally cross-reactive in short-term cultures, were associated with the highest disease severity in AIM, and displayed enhanced production of gamma interferon, a cytokine that greatly amplifies immune responses, thus frequently contributing to induction of immunopathology. Altogether, these data link heterologous immunity via CD8 T-cell cross-reactivity to CD8 T-cell repertoire selection, function, and resultant disease severity in a common and important human infection. In particular, it highlights for the first time a direct link between the TCR repertoire with pathogenesis and the diversity of outcomes upon pathogen encounter.

IMPORTANCE The pathogenic impact of immune responses that by chance cross-react to unrelated viruses has not been established in human infections. Here, we demonstrate that the severity of acute infectious mononucleosis (AIM), an Epstein-Barr virus (EBV)-induced disease prevalent in young adults but not children, is associated with increased frequencies of T cells cross-reactive to EBV and the commonly acquired influenza A virus (IAV). The T-cell receptor (TCR) repertoire and functions of these cross-reactive T cells differed between mild- and severe-AIM patients, most likely because these two groups of patients had selected different memory TCR repertoires in response to IAV infections encountered earlier. This heterologous immunity may explain variability in disease outcome and why young adults with more-developed IAV-specific memory T-cell pools have more-severe disease than children, who have less-developed memory pools. This study provides a new framework for

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understanding the role of heterologous immunity in human health and disease and highlights an important developing field examining the role of T-cell repertoires in the mediation of immunopathology.

KEYWORDS CD8, Epstein-Barr virus, TCR repertoire, cross-reactive, heterologous immunity, immune memory, influenza

Over 95% of the world's population acquires a persistent infection with Epstein-Barr virus (EBV) before the age of 30. The vast majority of acute EBV infections occur in childhood and are essentially asymptomatic or unremarkable (1, 2). However, 30 to 37% of U.S. college freshmen are EBV seronegative (3). Half of these EBV-susceptible individuals acquire EBV infection in the first 2 years of college, with most (60 to 77%) displaying symptoms of acute infectious mononucleosis (AIM) (3, 4). AIM is associated with a massive CD8 T-cell expansion; symptoms can vary greatly in severity from a mild short influenza-like illness to a more severe syndrome with sore throat, lymphadenopathy, splenomegaly, hepatomegaly, and debilitating fatigue lasting months (1, 2). A rare 5% of the population appears never to acquire the infection and remains EBV seronegative (5). Severe illness requiring hospitalization has been reported in individuals who acquire primary EBV infection late in life (6). Persistent EBV infection is also associated with the development of certain malignancies, including nasopharyngeal carcinoma and Burkitt's lymphoma (2) or autoimmune disorders such as multiple sclerosis (MS) (7).

The exact mechanisms that lead to induction of AIM are still very poorly understood. Most prior research suggests that EBV-specific CD8 T-cell responses are robustly expanded and may contribute to immunopathology in AIM; during the persistent phase of infection, EBV-specific CD8 T cells appear to be important in the control of viral replication (1, 2, 8–10). However, available data also suggest that EBV employs multiple mechanisms to evade the host immune response, and this may be critical to its ability to silently infect most of the population and establish latency in memory B cells (11, 12). For instance, EBV does not induce a strong type 1 interferon (IFN) response (13, 14); EBV also encodes an immunosuppressant interleukin-10 (IL-10) homologue within its genome (11, 12).

Here, we postulate that in some individuals, changes in the CD8 T-cell repertoire resulting from prior unrelated infections results in AIM immunopathology. Over the course of an individual's life, encounters with various antigens leave imprints on the immune system that affect innate and adaptive immune responses in subsequent infections. These heterologous effects on immunity may be beneficial or harmful (15–22). Evidence for heterologous immunity mediated via T-cell cross-reactivity, even to unrelated viruses, and its impact on disease outcome in both mouse and human studies is continuing to increase (23–28). Mouse models show that T-cell cross-reactivity can change patterns of T-cell immunodominance (24), lead to the generation of narrowly focused T-cell repertoires and T-cell escape viral mutants (29), and sometimes confer a level of beneficial protective immunity impacting the difference between life and death (17, 30, 31) but at other times be detrimental, leading to more-severe disease with substantially altered pathology (17, 26, 32, 33).

While mechanistic studies of humans are difficult to perform, AIM provides a system amenable to mechanistic analyses. We and others have described heterologous immunity to three viruses that infect humans (influenza A virus [IAV], hepatitis C virus, and dengue virus) (34, 35) and more recently between dengue virus and Zika virus (36). IAV, the cause of influenza, is an important human pathogen, and "heterotypic" or protective heterologous immunity has been documented via both CD4 and CD8 T-cell cross-reactivity to IAV strains (37–39). *Mycobacterium bovis* BCG, live polio, and measles vaccines have been reported to decrease death due to unrelated pathogens in developing countries (40). Intriguingly, children vaccinated with BCG also had a 40% lower incidence of atopy (41). Detrimental heterologous immunity may help explain why young adults, who have complex, large, potentially cross-reactive memory T-cell pools, commonly get more severely ill with infections like those due to EBV, cytomegalovirus

(CMV), varicella-zoster virus, and mumps virus, which normally are mild in younger children (42).

This study highlights the importance of virus-specific CD8 T-cell receptor (TCR) repertoires in the mediation of heterologous immunity. TCR sequence diversity is thought to enhance the surveillance efficiency of CD8 T cells and has been associated with improved viral control and reduced viral escape (34, 35, 37). We recently used TCR sequences to relate structural interactions between peptide-major histocompatibility complex (MHC) complexes and TCRs to the selection of TCR repertoires and the functional consequences of these interactions in response to the HLA-A2-restricted influenza virus A-M1₅₈ (IAV-M1) epitope (43). CD8 TCR repertoires in response to common viruses (IAV, CMV) are highly diverse and individualized; this is often referred to as the “private specificity” of TCR repertoires. However, “public” clonotypes, which are defined by the use of the same V, J, or CDR3 amino acid sequences in many individuals, are favored for expansion, likely because of selection for optimal structural interactions (44–46). Our recent results (43) suggest that antigen-specific TCR repertoires have evolved “focused diversity,” i.e., public clonotypes with highly diverse private responses, to provide the ability to rapidly recognize their antigen, while retaining flexibility should the antigen mutate or to assist in rapid responses to a new cross-reactive pathogen. Although there are some recent limited reports of the TCR repertoire being linked to disease (47), the role specific TCRs play in the mediation of T-cell functional responses and disease outcome is still poorly understood.

Some AIM patients and healthy persistently EBV-infected donors have HLA-A2.01-restricted CD8 T-cell responses cross-reactive to an immunodominant and highly conserved IAV-encoded M1₅₈ epitope (IAV-M1) and two EBV-encoded epitopes, EBV BMLF1₂₈₀ (EBV-BM) and EBV-BLRF1₁₀₉ (EBV-BR) (48, 49). We have also recently detected unique, functionally IAV-M1/EBV-BM cross-reactive oligoclonal CD8 TCR repertoires in five rare individuals who remain EBV seronegative (MA-EBV-SN) into their 4th decade of life, suggesting that cross-reactive CD8 T cells may protect from EBV infection (5). These studies provide a strong rationale for further examination of the potential role of both virus-specific and cross-reactive TCR repertoires in the mediation of clinical outcomes upon pathogen exposure. Mouse models of heterologous immunity have shown that the same epitope cross-reactive response can either be protective or induce immunopathology, depending on the individual’s history of infection and TCR repertoire (24, 49–54). Here we sought to determine if there is a correlation between the expansion of IAV-M1/EBV-BM cross-reactive T-cell responses and the severity of AIM during EBV infection. In addition, if these memory IAV-M1 cross-reactive responses had TCR repertoires different from those of seropositive persistently EBV-infected healthy donors (HD-SP) and if they differed between mild- and severe-AIM patients, this would be strong evidence that prior selection of cross-reactive IAV-M1 memory TCR repertoires influences the severity of AIM. Our hypothesis is that following infection of an immune host with a heterologous virus, cross-reactive T-cell responses, when present, are selectively expanded and impact the outcome of disease due to the heterologous virus. Thus, these studies have systematically examined whether CD8 T-cell cross-reactivity between an individual’s memory responses to IAV and EBV lytic antigens plays a role in the modification of antigen-specific CD8 population frequencies, function, and TCR repertoire and whether this correlates with disease outcome during AIM.

RESULTS

Characteristics of the study populations. Over a 10-year period, we enrolled 32 AIM patients and 17 healthy persistently EBV-infected seropositive donors (HD-SP). In both groups, the median age was 20 years and there was equal representation of males and females (see Table S1 in the supplemental material). Because the goal of this study was to determine the role of cross-reactive CD8 T cells in the mediation of AIM severity and because the clinical presentation of AIM varied greatly between patients, we developed a strategy to score disease severity. Adenopathy, the most common sign of AIM on physical examination (our unpublished data), directly correlated (Fig. S3, graph

i) with the degree of expansion of atypical lymphocytes, i.e., large granular lymphocytes documented on the peripheral blood smear and used as one of the diagnostic criteria for AIM. An inversion of the CD4/CD8 ratio in peripheral blood mononuclear cells (PBMCs) has long been associated with acute viral infection because of the large expansion of CD8 T cells, another pathognomic finding in AIM. We found that the CD4/CD8 ratio inversely correlated with the percentage of atypical lymphocytes (Fig. S3, graph ii). Thus, for some of the correlation studies, we used the percentage of atypical lymphocytes as a direct and quantitative measurement of AIM severity. However, in order to actually stratify each patient into the mild- or severe-AIM group, we scored AIM severity by using these same three direct measurements of AIM pathology, including the percentage of atypical lymphocytes in peripheral blood, the level of lymphadenopathy as scored by the same research nurse, and the CD4/CD8 ratio in PBMCs (details are in Materials and Methods) (Table S1). On the basis of this scoring strategy, AIM patients could be stratified into two groups, with severe-AIM patients having a significantly (Student *t* test) higher adenopathy score (6.5 ± 0.6 versus 3.3 ± 0.8 ; $n = 15$ or 16 ; $P = 0.01$) and percentage of atypical lymphocytes (44 ± 4.2 versus 23 ± 3.8 ; $n = 15$ or 16 ; $P = 0.002$) and a lower CD4/CD8 ratio (0.6 ± 0.08 versus 1.4 ± 0.1 ; $n = 15$ or 16 ; $P = 0.001$) than mild-AIM patients.

Evidence of increased IAV/EBV CD8 T-cell cross-reactivity in severe-AIM patients by tetramer costaining directly *ex vivo* in PBMCs. CD8 T-cell cross-reactivity can be complex and is easiest to demonstrate if costaining with two tetramers is successful, usually when the two epitopes have similar avidity to the same TCR (29, 48, 49). However, cross-reactive epitopes may not share sequence identity and weaker affinity for one of the ligands may, because of competition, reduce tetramer binding, as has been reported for MHC class I cross-reactive ligands in autoimmune diseases and tumor studies (55–57).

Costaining of CD8 T cells with epitope-specific tetramers is the most direct way to demonstrate cross-reactivity. Representative fluorescence-activated cell sorter (FACS) plots from patients in both severity groups demonstrate higher frequencies of circulating total IAV-M1 (without tetramer costaining), cross-reactive IAV-M1+EBV-BM, and cross-reactive IAV-M1+EBV-BR tetramer⁺ CD8 T cells in a representative severe-AIM patient than in a mild-AIM patient or a control HD-SP (Fig. 1a). These cross-reactive responses usually peaked at visit 1 or 2 and then declined at subsequent visits, along with the total EBV-BM- and EBV-BR-specific responses. In contrast, there was no evidence of cross-reactive responses to CMV pp65 by tetramer costaining during AIM in CMV-seropositive donors (data not shown). However, the tetramer costaining method has to be carefully controlled, as in some cases one tetramer binds with higher affinity and blocks the binding of the other (Fig. 1b and c) (49). This problem was particularly evident in the severe-AIM patient group, where there was significantly more blockade of the cross-reactive IAV-M1 tetramer binding by EBV-BM or EBV-BR tetramers *ex vivo* than staining in mild-AIM patients (Fig. 1b and c; Fig. S2a). T cells can also cross-react to EBV-BM and EBV-BR, and EBV-BM tetramer binding was also blocked by EBV-BR tetramer in some severe-AIM patients (Fig. 1c; Fig. S2a). Epitope-specific blockage of tetramer staining was also present in some AIM patient short-term cultures (Fig. S2b). Because of these issues with tetramer blocking affecting the accuracy of tetramer frequency determination, when we refer to IAV-M1, EBV-BM, or EBV-BR tetramer⁺ frequencies, we are referring to the total population by using data from the single tetramer staining frequencies (these frequencies will thus include the cross-reactive populations). Thus, ironically and very inconveniently, the blockade of the binding of one tetramer by the presence of a second tetramer is also further evidence of cross-reactivity.

Directly *ex vivo* in PBMCs, only IAV-M1 and IAV-M1+EBV-BM tetramer⁺ CD8 T cells strongly correlate with AIM severity and predict an increased relative risk of severe AIM. Significantly increased CD8 T-cell responses to total EBV-BM, to total EBV-BR, and to total IAV-M1 in AIM patients were detected directly *ex vivo* by tetramer staining (Fig. 2). Twenty-four-fold and 185-fold increases in the numbers of EBV-BM and

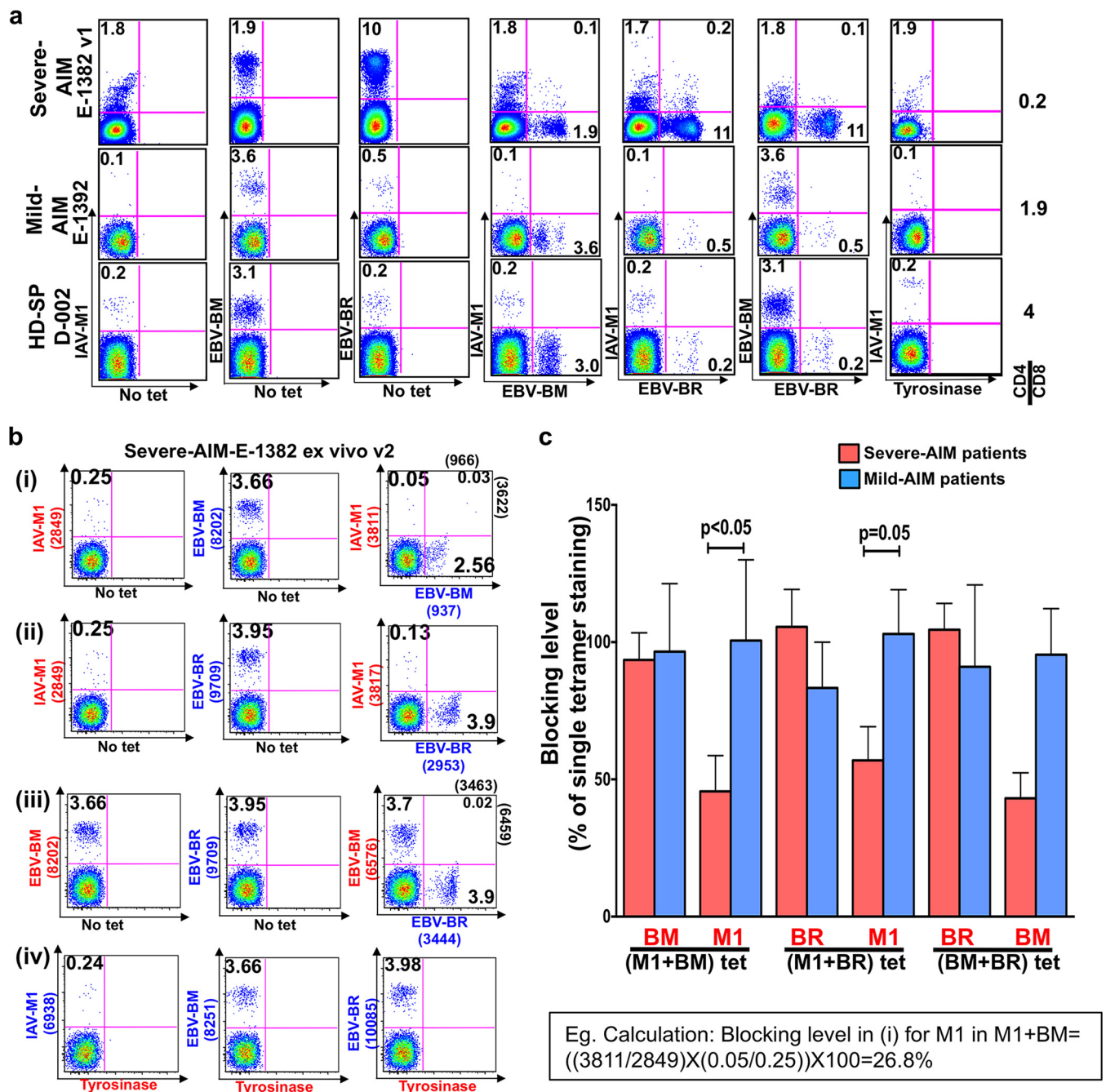


FIG 1 Evidence of increased IAV/EBV CD8 T-cell cross-reactivity in PBMCs of severe-AIM patients by tetramer costaining directly *ex vivo*. (a) One way to determine the frequency of cross-reactive CD8 T cells in peripheral blood sorted CD8 T cells is costaining of cells with different tetramer pairs as shown in this representative example, which shows that during the acute phase of AIM at visit 1 (v1), a severe-AIM donor (E-1382) has more IAV-M1+EBV-BM tetramer⁺ CD8 T cells than a mild-AIM donor (E-1392) and HD-SP (D-002). (b) This same severe-AIM patient at v2 showed a competitive reaction and mutual tetramer blocking of IAV-M1 and EBV-BM tetramer binding upon costaining with these two tetramers. Costaining with IAV-M1 and EBV-BR tetramers did not result in double-tetramer-positive cells, but the level of IAV-M1 tetramer⁺ cells declined compared to that of IAV-M1 single-tetramer-staining cells. There was no blockade upon costaining with EBV-BM and EBV-BR or IAV-M1 and control tyrosinase₃₆₉₋₃₇₇ tetramers. In these studies, the exact same tetramers were used for the single-color control and double-tetramer staining. The values in parentheses are the MFIs of the populations indicated. (c) Significant IAV-M1-specific tetramer blockade by EBV-BM-specific tetramer and EBV-BR-specific tetramer was detected *ex vivo* in severe-AIM patients ($n = 11$) compared to mild-AIM patients ($n = 7$). On the x axis, the red text indicates the tetramer-specific response that was being blocked by the other tetramer. Below the line is the double-tetramer combination used for costaining. The estimated blocking level for each tetramer was calculated by the formula [(costained tetramer A MFI/alone tetramer A MFI) \times (costained tetramer A%/alone tetramer A%)] \times 100. The gating strategies used are shown in Fig. S1.

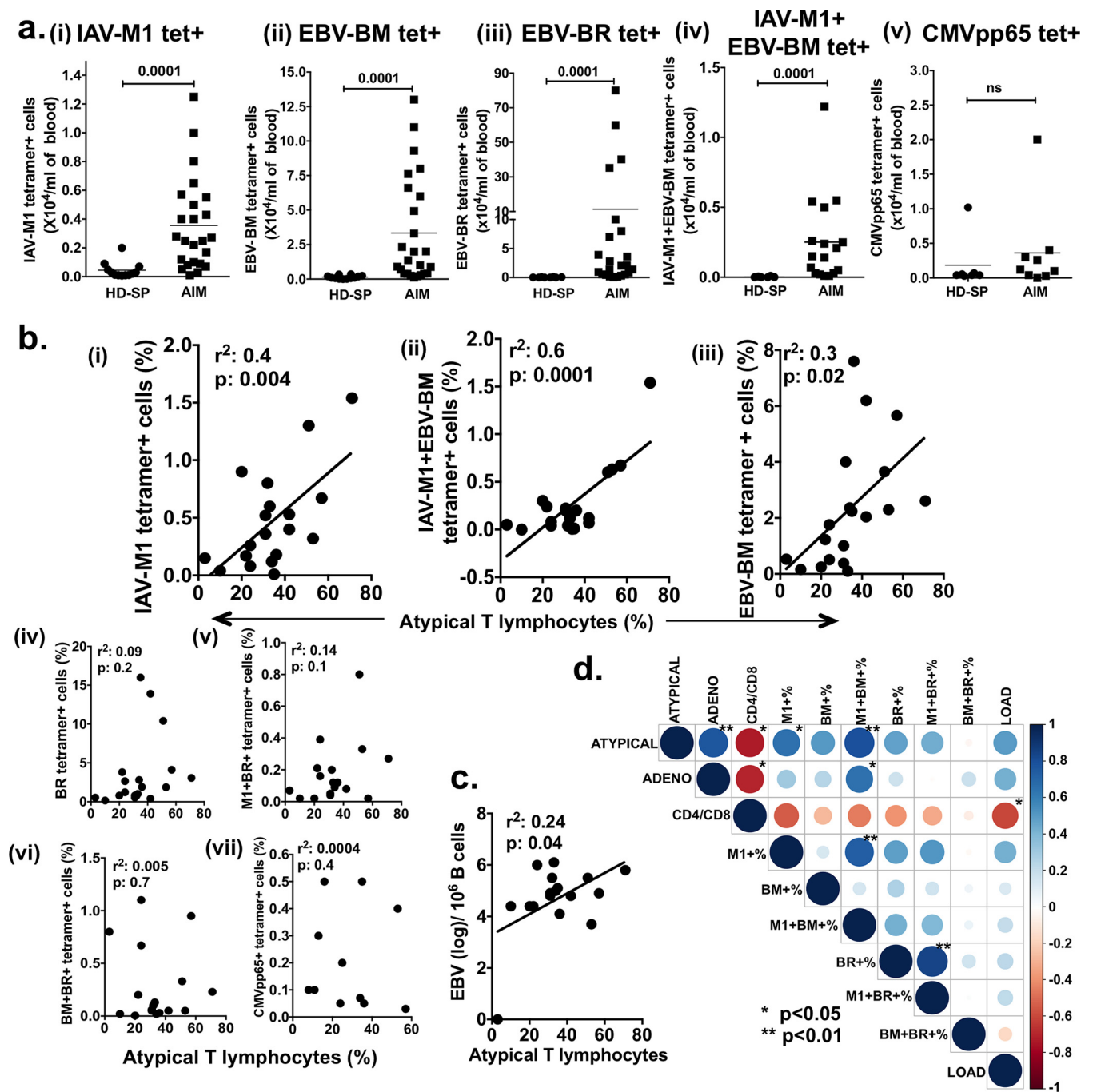


FIG 2 Only total IAV-M1 and IAV-M1+EBV-BM tetramer⁺ CD8 T cell levels in PBMCs analyzed *ex vivo* strongly correlate with AIM severity and predict the increased relative risk of severe AIM. (a) The average numbers of tetramer⁺ IAV-M1 (M1) (i), EBV-BM (BM) (ii) EBV-BR (BR) (iii) and IAV-M1+EBV-BM (M1+BM) (iv), but not CMV pp65-specific (v), CD8 T cells/ml of peripheral blood were significantly higher in AIM patients at the peak of their CD8 response than in healthy persistently infected EBV-seropositive donors (HD-SP) ($n = 8$ to 12) (Mann-Whitney U test). ns, not significant. (b) During the course of AIM, the peak (highest) frequency of tetramer⁺ IAV-M1 (i), IAV-M1+EBV-BM (ii), and EBV-BM (iii) CD8 T cells *ex vivo* directly correlated with the percentage of atypical lymphocytes; the peak frequency of tetramer⁺ EBV-BR (iv), IAV-M1+EBV-BR (v), EBV-BM+EBV-BR (vi), and CMV pp65 (vii) CD8 T cells *ex vivo* did not correlate with the percentage of atypical lymphocytes ($n = 17$ to 19 AIM patients per analysis). (c) During the course of EBV infection, the peak (greatest) viral load (measured as the genome copy number per B cell) had a weak direct correlation with disease severity measured as the percentage of atypical lymphocytes. (d) Display of pairwise correlations between variables of interest computed in a correlation matrix by using the Pearson correlation coefficient (the P values shown are adjusted for the number of multiple variant comparisons) and then graphically displayed as a matrix by using the corplot R package with dark blue as the most positive correlation coefficient of 1 and dark red an inverse correlation coefficient of -1 . Relative-risk analyses are shown in Table S2.

EBV-BR tetramer⁺ cells/ml of blood, respectively, were observed, along with 10-fold and 25-fold increases in cross-reactive IAV-M1 and IAV-M1+EBV-BM tetramer⁺ cells/ml compared to those in HD-SP (Fig. 2a). This did not appear to be a nonspecific or bystander expansion of all memory cells, as an increase in the number of CMV pp65 tetramer⁺ cells/ml was not observed in CMV-seropositive AIM patients (Fig. 2a, graph v).

We then questioned whether there was any evidence of a potential role for these cross-reactive IAV-M1 tetramer⁺ cells in the mediation of AIM severity. Interestingly, only some of these IAV and EBV epitope-specific CD8 T-cell responses correlated with disease severity. Only the *ex vivo* peak (highest) frequencies of total IAV-M1 and the cross-reactive double IAV-M1+EBV-BM tetramer⁺ subset, but not the IAV-M1+EBV-BR tetramer⁺ subset, directly correlated with the severity of AIM measured as the percentage of atypical lymphocytes (Fig. 2b). Interestingly, of the EBV-specific responses, only the total EBV-BM tetramer⁺ frequencies weakly correlated with AIM severity, but not the other early dominant epitope-specific response, EBV-BR or cross-reactive EBV-BM and EBV-BR tetramer⁺ cells. There was also no correlation between another memory population, CMV pp65 tetramer⁺ frequency and AIM severity (Fig. 2b). There was a weak correlation between AIM severity and the peak EBV load (Fig. 2c). It should also be noted that multivariate analysis of the peak total IAV-M1, EBV-BM, and EBV-BR tetramer⁺ frequencies *ex vivo* suggested that the total IAV-M1 tetramer⁺ frequencies were equivalently expanded within the mild- and severe-AIM groups during AIM to the EBV epitope responses, except in one case; the EBV-BR tetramer⁺ frequency was greater than the IAV-M1 tetramer frequency in the severe-AIM group (one-way analysis of variance [ANOVA] with Tukey's multiple-comparison test, $P < 0.04$). However, the display of pairwise correlations between all of these variables of interest, when computed in a correlation matrix by using the Pearson correlation coefficient (Fig. 2d) and multivariate analysis, suggests that there is something unique about the total IAV-M1 tetramer⁺ response during AIM and in particular the double IAV-M1+EBV-BM tetramer⁺ cross-reactive response, which may drive AIM severity. Only these two factors significantly correlated with disease severity after adjustment for multivariate comparisons. It is possible, if not likely, that the majority of the total IAV-M1 tetramer⁺ cells in AIM were cross-reactive with EBV-BM, as their frequency directly correlated with the IAV-M1+EBV-BM tetramer⁺ frequency (Fig. 2d). In contrast, the frequency of cross-reactive IAV-M1+EBV-BR tetramer⁺ cells correlated with that of EBV-BR tetramer⁺ cells (Fig. 2d). In fact, relative-risk analyses also revealed that only total IAV-M1 tetramer⁺ (if $\geq 0.36\%$, relative risk = 4.9, odds ratio = 14, $P < 0.05$; Fisher's exact test) and IAV-M1+EBV-BM tetramer⁺ (if $\geq 0.1\%$, relative risk = 5.8, odds ratio = 18.67, $P < 0.02$) peak frequencies (usually at visit 1 or 2) could predict an increased risk of developing severe AIM (Table S2). As in our highly controlled mouse studies of heterologous immunity (33, 50), these cross-reactive IAV-M1-specific CD8 T-cell responses that expanded during AIM were likely due to reactivated memory cells rather than *de novo* new naive responses, as all of these patients were IAV immune. This is further supported by the observation that, in naive cord blood, IAV-M1-specific CD8 T cells do not proliferate in response to EBV-BM or EBV-BR peptide stimulation (5). On the basis of these studies, we propose a link between IAV-M1 and EBV-BM cross-reactive T cells and AIM severity.

Directly *ex vivo* in PBMCs, the mean frequency of total IAV-M1 and IAV-M1+EBV-BM tetramer⁺ CD8 T cells was increased in the severe-AIM group. Despite individual variation in EBV-specific responses, all AIM patients had significantly higher mean frequencies of circulating total IAV-M1 (5-fold), EBV-BM (5-fold), and EBV-BR (15-fold) tetramer⁺ cells, measured directly *ex vivo* in PBMCs than did HD-SP (Fig. 3a). Interestingly, as in the correlation studies, when the donors were segregated into mild- and severe-AIM groups, the mean frequencies of total IAV-M1 or EBV-BM tetramer⁺ cells were 2.3- and 2.4-fold significantly greater, respectively, in severe-AIM than in mild-AIM patients (Fig. 3b). Although the mean frequency of total EBV-BR tetramer⁺ cells was higher in the severe-AIM group, the difference did not reach

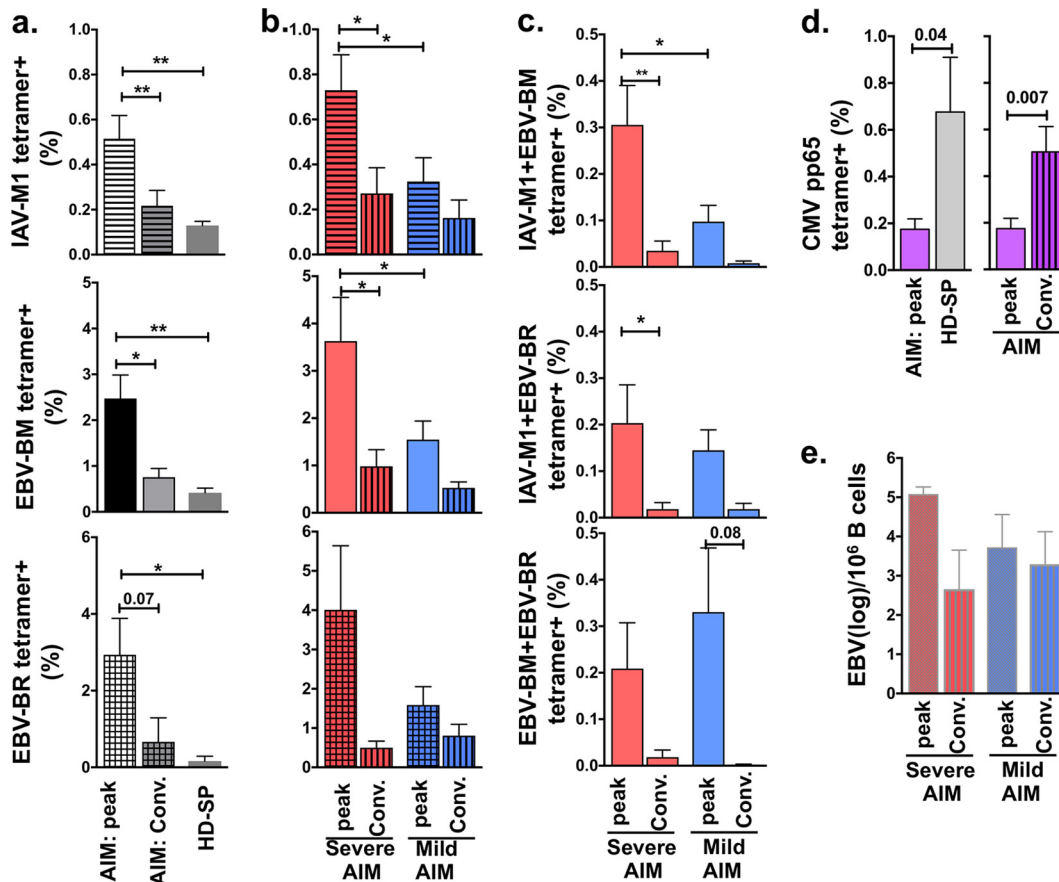


FIG 3 PBMCs in patients with severe AIM had a greater mean frequency of total IAV-M1, EBV-BM, and IAV-M1+EBV-BM tetramer⁺ CD8 T cells than mild-AIM patients (when analyzed *ex vivo*). (a) All AIM patients had a significantly higher mean peak frequency of total IAV-M1-, EBV-BM-, and EBV-BR-specific tetramer⁺ CD8 T cells than healthy persistently infected EBV-seropositive donors (HD-SP). (b) When patients were categorized into two groups on the basis of disease severity (see Materials and Methods), severe-AIM patients had a significantly higher mean peak frequency of total IAV-M1 and EBV-BM tetramer⁺ cells, but not EBV-BR, directly *ex vivo* in their PBMCs than mild-AIM patients ($n = 8$ to 22 donors per group). The *ex vivo* mean frequency of total IAV-M1 and EBV-BM tetramer⁺ cells significantly decreased from the peak to the convalescent (conv) phase in the severe-AIM group ($n = 6$ to 12 donors per group). (c) The mean peak frequency of IAV-M1+EBV-BM tetramer⁺ cells in severe-AIM patients was higher than that in mild-AIM patients but not that in the other two cross-reactive populations. (d) In CMV-seropositive donors, CMV pp65 tetramer⁺ CD8 T cells were lower in AIM patients than in HD-SP and higher in the convalescent phase than the peak CD8 T-cell responses during AIM (AIM patients, $n = 11$; convalescent [Conv.] AIM patients, $n = 8$; HD-SP, $n = 11$). (e) There was no significant difference in the mean peak or convalescent-phase EBV load (measured as the genome copy number [log] per 10⁶ B cells) between severe- and mild-AIM patients. The Student *t* test was used to compare two groups, and one-way ANOVA with Sidak's multiple-comparison test was used to compare more than two. Severe-AIM groups, red; mild-AIM groups, blue. *, $P < 0.05$; **, $P < 0.01$.

statistical significance, mostly likely because of great variation in frequency, as some severe-AIM patients had a percentage of EBV-BR tetramer⁺ cells as low as 0.4%. Differences in frequencies between patient groups were not detected in the convalescent phase 6 to 12 months after the acute phase of AIM (Fig. 3b). This was most likely due to a significant 2.6- and 3.7-fold decrease in the mean frequency of IAV-M1 and EBV-BM tetramer⁺ cells, respectively, in severe-AIM patients from the acute to the convalescent phase of infection (Fig. 3b). In mild-AIM patients, the expansion of all three epitope-specific responses during the acute phase was so moderate that it did not differ from that in the convalescent phase.

There also was a selective significant 3-fold greater mean peak frequency of cross-reactive IAV-M1+EBV-BM tetramer⁺ cells in severe-AIM patients than in mild-AIM patients, while the frequencies of the other two cross-reactive populations, IAV-M1+EBV-BR and EBV-BM+EBV-BR tetramer⁺ cells, were similarly increased in both patient groups (Fig. 3c). As mentioned above, in young CMV-seropositive donors during

AIM, there was no increase in CMV pp65 tetramer⁺ CD8 cells (Fig. 3d), which were generally not cross-reactive with EBV lytic epitopes, as assessed by double tetramer binding. In fact, CMV pp65 tetramer⁺ cells were diluted out by the EBV-specific response, resulting in a significantly lower frequency in the acute phase of AIM than in the convalescent phase or in HD-SP. As in the multivariate correlation analyses, when the donors were separated into mild- and severe-AIM groups, there was no significant difference in the mean EBV load measured as the genome copy number in B cells during the acute or convalescent phase (Fig. 3e). These results further support the proposed selective link between IAV-M1 and EBV-BM cross-reactive T cells and AIM severity. In subsequent studies, we examined whether the TCR repertoire and associated functional responses in this cross-reactive population could mechanistically account for the difference in disease severity between mild- and severe-AIM patients.

Directly *ex vivo* in PBMCs, mild- and severe-AIM patients were shown to use different TCR V β repertoires in their total cross-reactive IAV-M1 tetramer⁺ cells, suggesting that the particular TCR repertoire played a role in disease severity, as these memory T cells were present in each donor prior to acute EBV infection. TCR repertoires of IAV-M1 tetramer⁺ cells in HD-SP and even young EBV-seronegative donors prior to infection with EBV are similar, with distinct characteristics. Studies have shown that they exhibit “focused diversity” in that they are highly diverse, differing between individuals, but strongly focused on TCR V β 19 use and particular CDR3 motifs (5, 58). Therefore, if IAV-M1-specific TCR repertoires changed during AIM, this would be consistent with selective expansion of cross-reactive TCR repertoires rather than bystander activation of all IAV-M1 memory cells. Also, if the IAV-M1 TCR repertoire differed between mild- and severe-AIM patients, this would also suggest that they had different IAV-M1 TCR repertoires cross-reactive with EBV prior to being infected with EBV. This would be consistent with different IAV-M1 cross-reactive TCR repertoires driving different disease severities during AIM rather than EBV infection just activating IAV-M1 cross-reactive cells randomly. Therefore, we next questioned whether the IAV-M1-specific TCR repertoires of the three donor groups differed. This was determined by direct costaining with IAV-M1 tetramer and V β -specific monoclonal antibodies (MAbs) on *ex vivo* sorted CD8 T cells from fresh PBMCs. Using the Simpson diversity index (SDI) (59), we found that AIM patients (mean SDI, 0.7 ± 0.09 ; $n = 19$) had significantly more diverse IAV-M1-specific TCR V β repertoires than HD-SP (mean SDI, 0.2 ± 0.08 ; $n = 8$) ($P < 0.0001$, Student *t* test) (Fig. 4a). The TCR V β repertoire of IAV-M1-specific T cells differed between AIM patients and HD-SP. Consistent with previous reports (58, 60), TCR V β 19, the most commonly used IAV-M1-specific TCR V β type in HD-SP ($56.5\% \pm 5.6\%$, $n = 18$), significantly decreased in AIM patients ($20.4\% \pm 5.2\%$, $n = 18$, $P < 0.0001$) (Fig. 4b, graph i). IAV-M1-specific TCR repertoires included multiple V β families, and V β use varied between HD-SP and AIM patients, as AIM patients preferentially used V β 20, -9, -2, and -29 during the acute phase of infection (Fig. 4b, part ii). The IAV-M1-specific TCR V β repertoire also significantly differed between mild- and severe-AIM patients. Severe-AIM patients ($n = 12$) preferentially used many V β types, including V β 20, -9, -28, -27, -6.2, and -4.1, but they only used V β 4.1 significantly more than the mild-AIM patients. The mild-AIM patients ($n = 8$) also used multiple V β types but preferentially used V β 29 and V β 2 more than severe-AIM patients did during infection (Fig. 4c). Since severe- and mild-AIM patients had unique IAV-M1-specific TCR V β use, we questioned whether the use of a particular TCR V β type directly correlated with AIM severity. Indeed, there was a direct correlation between IAV-M1-specific TCR V β 4.1 use and the peak frequency of atypical T lymphocytes, heralding severity of disease (Fig. 4d, part i). There was an inverse correlation between IAV-M1-specific TCR V β 2 use and the peak frequency of atypical T lymphocytes (Fig. 4d, part ii). Thus, the cross-reactive IAV-M1 TCR repertoire differed between mild- and severe-AIM patients, and in fact, disease severity correlated with specific TCR V β use within the cross-reactive IAV-M1-specific cells in AIM patients. These results are strongly supportive of the concept that the cross-reactive IAV-M1 TCR repertoire that exists in an individual prior to EBV infection determines disease severity during AIM.

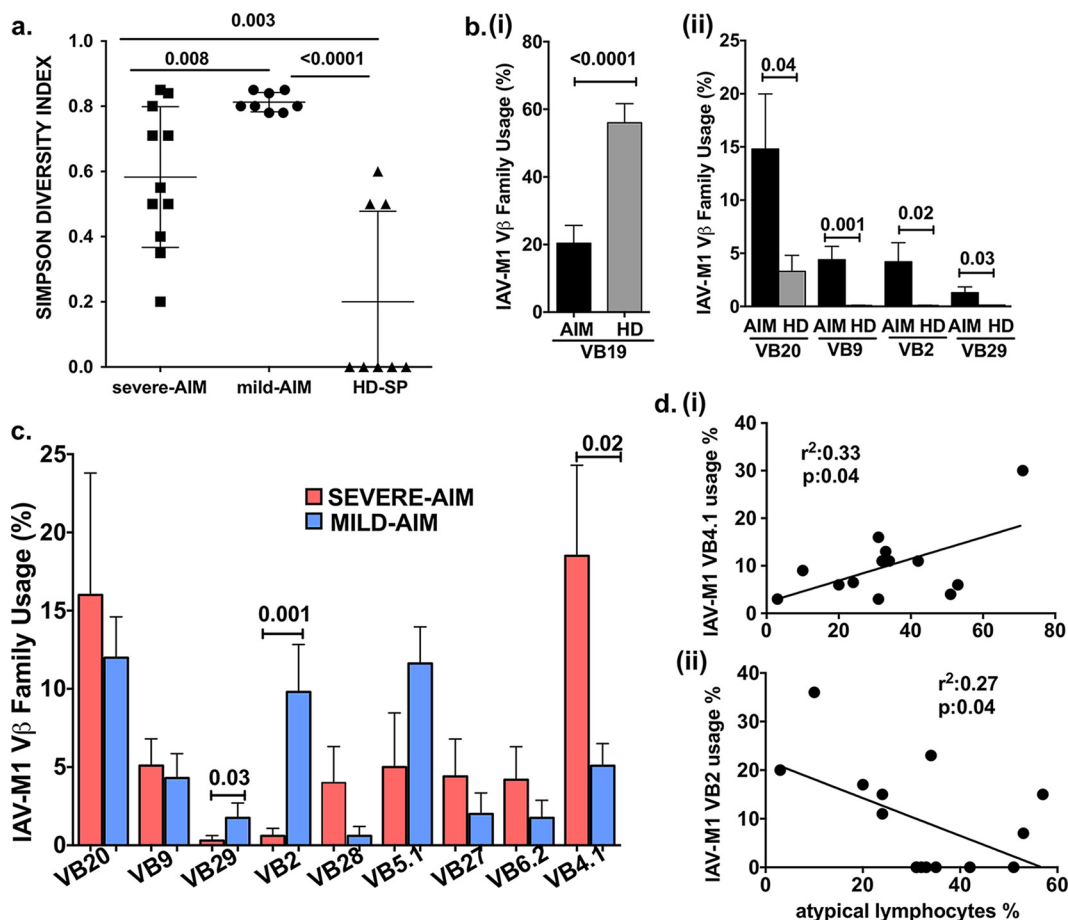


FIG 4 During AIM, patients with mild or severe disease have selected different TCR Vβ families in the expanding cross-reactive IAV-M1 tetramer⁺ cells (analyzed directly *ex vivo* in PBMCs). This suggests that each group had different cross-reactive TCR repertoires prior to acute EBV infection, with differential effects on disease severity. (a) Directly *ex vivo* sorted CD8 T cells from PBMCs were costained with IAV-M1 tetramer and Vβ-specific MAbs. Both severe-AIM ($n = 11$) and mild-AIM ($n = 8$) patients had more diverse IAV-M1-specific TCR Vβ family use than HD-SP ($n = 8$), as calculated by the SDI (see Materials and Methods). (b, part i) Decreased use of commonly used Vβ19 in the IAV-M1-specific TCR repertoire in AIM patients compared to that in HD-SP. (b, part ii) The Vβ repertoire of IAV-M1-specific T cells differed between AIM patients ($n = 19$) and HD-SP ($n = 18$), with increased use of Vβ20, -9, -2, and -29, which are more often associated with EBV-BM responses. (c) IAV-M1-specific TCR Vβ repertoires differed between mild- and severe-AIM patients. Severe-AIM patients ($n = 11$) preferentially used Vβ4.1, while mild-AIM patients ($n = 8$) preferentially used Vβ2 and Vβ29. (d, part i) Disease severity correlated directly with specific TCR Vβ use in AIM patients, suggesting that it plays a role in the mediation of disease severity. There was a direct correlation between the frequency of IAV-M1-specific TCR Vβ4.1 use and the peak frequency of atypical T lymphocytes during AIM. (d, part ii) There was an inverse correlation between the frequency of IAV-M1-specific TCR Vβ2 use and the peak frequency of atypical T lymphocytes. The TCR Vβ frequency of each donor is based on the mean of the first four visits (to enhance reproducibility), except for Vβ4.1, where it is based on the mean of all visits. The Student *t* test was used to compare two groups, and one-way ANOVA with Sidak's multiple-comparison test was used to compare more than two groups.

Differences in the functionality of IAV/EBV cross-reactive CD8 T cells in different donor groups determined by combining tetramer staining and ICS in short-term *in vitro* culture. There is evidence that tetramer staining does not always correlate with functional responses to peptide (61). Recent studies suggest that tetramer staining may underestimate the frequencies of antigen-specific cells (43, 44). Therefore, it was important to also conduct functional studies, such as measurement of proliferation or cytokine production in response to stimulation with each ligand (33, 48, 49). Studies have also suggested that TCR use may be linked to particular T-cell functions (62). We have previously observed that the CD8 T-cell populations that expand *in vitro* in short-term cultures reflect the characteristics, both the TCR repertoire and the activation state, of the *in vivo* cells within a donor (5, 43, 48, 49, 60). We thus questioned whether the cross-reactive TCR repertoires under study were associated with qualitative differences in functional responses, such as proliferation or cytokine

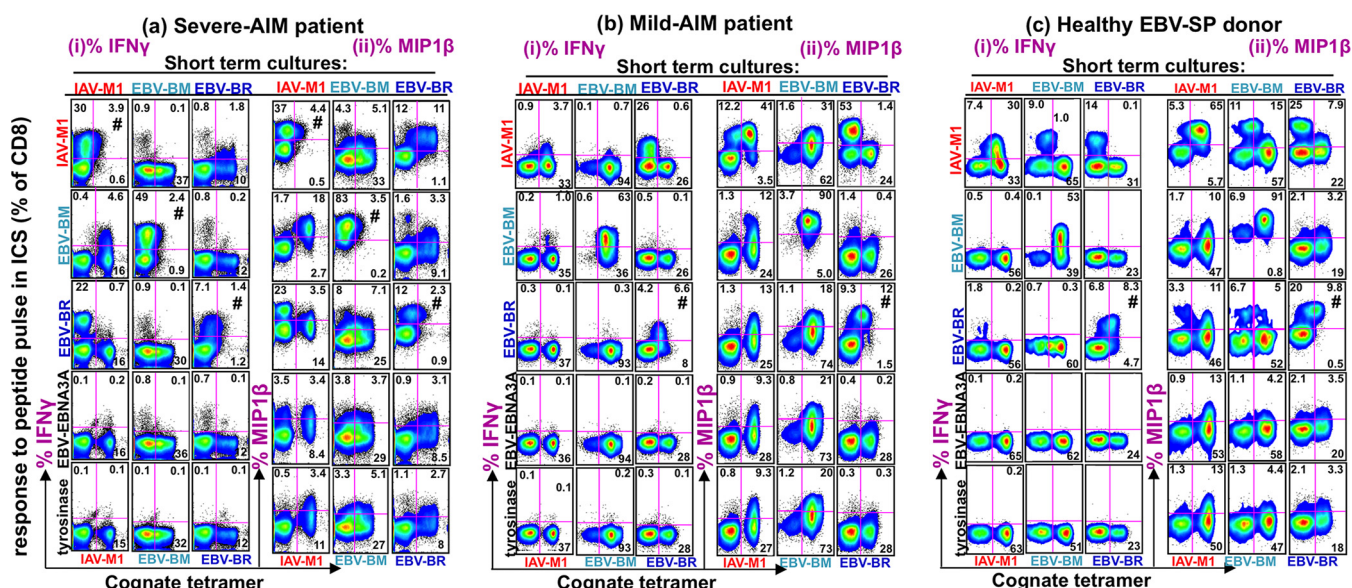


FIG 5 Representative examples of tetramer and intracellular cytokine costaining of CD8 T cells in short-term cultures suggesting that severe-AIM patients have the strongest functional cross-reactive responses. IAV-M1, EBV-BM, and EBV-BR peptide-stimulated short-term *in vitro* cultures generated from sorted CD8 T cells of representative severe-AIM (E-1302) (a) and mild-AIM (E-1392) (b) patients and an HD-SP (D-002) (c) were costained with cognate (same as the culture-stimulating peptide) tetramer and pulsed with cognate, cross-reactive, and control peptides (EBV latent epitope-specific peptide EBV-EBNA3A_{509–604} and self-peptide tyrosinase_{369–377}). IFN- γ (i) and MIP-1 β (ii) production was determined. The symbol # indicates that a cognate peptide pulse can result in such strong ligation of the TCR that it downregulates the TCR and thus the tetramer binding is hampered. (a) Representative severe-AIM patient who had more cross-reactive cytokine-producing cells (IFN- γ ⁺ or MIP-1 β ⁺) in all three cultures. IAV-M1 tetramer⁺ cells (in IAV-M1 culture) produced both IFN- γ and MIP-1 β in response to an EBV-BM peptide pulse, and EBV-BR tetramer⁺ cells (in EBV-BR culture) produced both IFN- γ and MIP-1 β in response to an IAV-M1 peptide pulse. (b) In the mild-AIM patient, there was generally a much lower frequency of cross-reactive responses and only IAV-M1 tetramer⁺ cells produced both IFN- γ and MIP-1 β in response to an EBV-BM peptide pulse. In the mild-AIM patient, the EBV-BR culture had a weaker type of cross-reactivity, as an IAV-M1 peptide pulse resulted in IFN- γ and MIP-1 β production in cells that were not BR tetramer⁺. (c) The HD-SP had even lower frequencies of cross-reactive responses than the mild-AIM patient, and they were predominantly of the weaker type.

production upon interaction with either ligand *in vitro*. To study the qualitative differences and assess their functional profiles, we questioned whether these cross-reactive IAV-M1 tetramer⁺ cells would proliferate in IAV-M1-, EBV-BM-, and EBV-BR-stimulated short-term cultures and whether they produced either IFN- γ or MIP-1 β in response to a peptide pulse. By combining cognate tetramer staining (tetramer specificity is the same as the stimulating peptide of the culture) with intracellular cytokine staining (ICS), we were able to determine if tetramer⁺ cells were making cytokines when pulsed with cross-reactive peptides (these are all other peptides except the peptide used to stimulate the culture, which can induce cytokines). Figure 5 shows examples of these combined tetramer-cytokine staining patterns of representative donors. Two types of cross-reactivity were observed, and the amount of each type of cross-reactivity present in any culture varied between donors. First, there could be a population of CD8 T cells with stronger, more obvious cross-reactivity where cognate tetramer⁺ cells made cytokines in response to a cross-reactive peptide pulse. The second population was evidence of weaker functional cross-reactivity, where CD8 T cells that had proliferated in response to the stimulating (cognate) peptide in culture did not bind the cognate tetramer but did make cytokines in response to cross-reactive peptide pulsing. We observed some differences between the representative donors from the three patient groups. The severe-AIM patient had the greatest frequency of cross-reactive IFN- γ -producing cells and the greatest number of different functionally cross-reactive populations between the EBV epitopes and IAV-M1 in all three cultures, resulting in IFN- γ production. This included the stronger cross-reactivity type, with four different cognate tetramer⁺ populations making IFN- γ upon a cross-reactive peptide pulse (IAV-M1 tet⁺/EBV-BM pulse, IAV-M1 tet⁺/EBV-BR pulse, EBV-BR tet⁺/IAV-M1 pulse, and EBV-BR tet⁺/EBV-BM pulse) (Fig. 5a). It also included the weaker cross-reactivity type with five different non-tetramer⁺ populations making IFN- γ in response

to a cross-reactive peptide pulse (IAV-M1 culture/EBV-BR pulse, EBV-BM culture/IAV-M1 pulse, EBV-BM culture/EBV-BR pulse, EBV-BR culture/IAV-M1 pulse, and EBV-BR culture/EBV-BM pulse). The cross-reactive peptide pulses induced MIP-1 β production even more often than IFN- γ (Fig. 5a), consistent with the fact that it is easier to induce MIP-1b with lower-avidity interactions (48). The representative mild-AIM patient and the HD-SP predominantly produced MIP-1 β more than IFN- γ upon a cross-reactive peptide pulse and had more of the weaker type of cross-reactivity. This might suggest that the difference in the IAV-M1 memory cross-reactive TCR repertoire prior to EBV infection in the two AIM patient groups (Fig. 4) results in the expansion of stronger affinity highly functional cross-reactive responses in the severe-AIM group upon EBV infection than in the mild-AIM group. Examination of functional cross-reactivity in these same samples by directly gating on the cognate tetramer⁺ cells and showing an overlay of IFN- γ or MIP-1 β histogram values for each peptide stimulation also demonstrated that the severe-AIM patient had a greater number of functionally cross-reactive responses between IAV-M1 and EBV-BM and -BR than the mild-AIM patient or HD-SP (Fig. S4). The histogram data also show that of the eight peptides tested, the cross-reactive responses were largely restricted to the IAV-M1, EBV-BM, and EBV-BR peptides, indicating high selectivity in this process. Overall, these representative data from each of the three patient groups suggest that there was greater proliferation of functionally cross-reactive cells, in particular IFN- γ -producing cells, in AIM patients than in HD-SP and more in severe-AIM patients than in mild-AIM patients. This conclusion is further supported by a statistical analysis of all of the subjects tested, as demonstrated below. In the next two sections, we summarize our systematic statistical analyses of the qualitative functional differences in both cell proliferation and cytokine production in the cross-reactive and cognate populations among all three donor groups that may help account for the differences in disease severity. However, using tetramer staining combined with ICS assays has certain drawbacks, as the peptide pulse in the ICS can lead to downmodulation of the TCR (e.g., Fig. 5a, IAV-M1 cognate tetramer with IAV-M1 pulse) and thus dramatically decrease tetramer binding and confound interpretation of the data. Therefore, to actually determine if there were significant functional differences in proliferation of the cross-reactive and cognate responses in the three patient groups in the different cell cultures, we examined tetramer frequency without peptide pulsing in the short-term *in vitro* cultures. To determine if there were significant functional differences in cytokine production of the cross-reactive and cognate responses in the three patient groups in the different cell cultures, we quantified the number of cells producing cytokines in response to cognate and a cross-reactive peptide pulse (without tetramer) and determined the frequencies of double-cytokine producers (IFN- γ ⁺ MIP-1 β ⁺) and single-cytokine producers (MIP-1 β ⁺).

Summary analyses of cross-reactive CD8 T-cell proliferation by tetramer frequency: unique profiles in donor groups with severe-AIM patients having the greatest proliferation of IAV/EBV cross-reactive CD8 T cells *in vitro*. We examined if the proliferative capacity of the cognate (same peptide used to grow the culture) and cross-reactive CD8 T cells differed among the three different donor groups by determining the cognate and cross-reactive tetramer frequency profiles in IAV-M1, EBV-BM, and EBV-BR peptide-stimulated short-term cultures. The proliferation of IAV-M1-, EBV-BM-, and EBV-BR-specific cells in each culture was determined by costaining with pairs of tetramers (representative tetramer staining in cultures Fig. S2b). As previously reported (48), in the tetramer costaining studies, we observed two types of cross-reactive cells, those that costained with two tetramers and those that stained with only one tetramer but expanded *in vitro* in response to the cross-reactive peptide (also see Fig. S2b). In each culture, we determined the tetramer frequency of cognate (same as the culture) (Fig. 6a and b) and cross-reactive cells that were either single tetramer⁺ (Fig. 6a and b; Fig. S5a) or double tetramer⁺ (Fig. 6c and d; Fig. S5b). The cognate EBV-BM cells *in vitro* proliferated as well in AIM patients as in HD-SP, while cognate EBV-BR and IAV-M1 did not proliferate as well in severe-AIM patients as in HD-SP. Instead, the cross-reactive IAV-M1 responses dominated in culture, particularly in cells

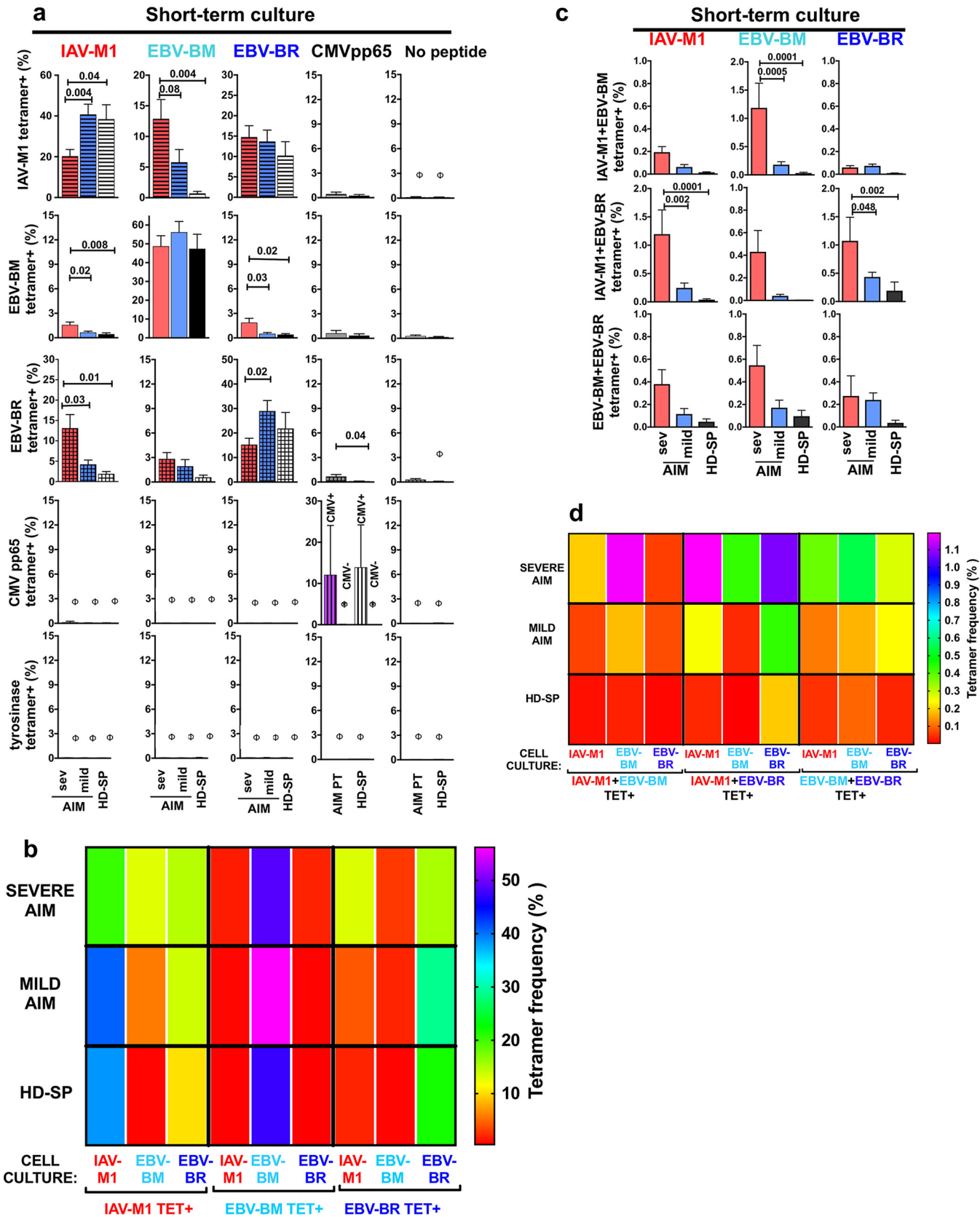


FIG 6 Severe-AIM patients have the greatest proliferation of IAV/EBV cross-reactive CD8 T cells in short-term *in vitro* cultures. In addition, each patient group maintains unique identifiable cognate and cross-reactive CD8 T-cell proliferation profiles upon peptide stimulation in short-term *in vitro* cultures, which are representative of their *in vivo* CD8 T-cell repertoires. The proliferation of IAV-M1-, EBV-BM-, and EBV-BR-specific cells in each culture was determined by

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derived from severe-AIM patients. The cognate EBV-BR-specific cells may be in a more functionally exhausted state *in vivo*, thus proliferating poorly *in vitro* in severe-AIM patients. Each donor group had a unique profile of cognate and single cross-reactive IAV/EBV-specific CD8 T-cell proliferation, as demonstrated in the heat map display with multivariate analyses (Fig. 6b; Fig. S5).

There was also an increased expansion of IAV/EBV double tetramer⁺ cross-reactive cells in severe-AIM patients (Fig. 6c and d). There was a significantly higher frequency of three cross-reactive populations, IAV-M1+EBV-BM tet⁺ cells in BM-stimulated cultures and IAV-M1+EBV-BR tet⁺ cells in both IAV-M1- and EBV-BR-stimulated cultures, in severe-AIM patients than in mild-AIM patients or HD-SP. Severe-AIM patients had a unique specific profile of double tetramer⁺ cross-reactive IAV/EBV-specific CD8 T-cell proliferation, as demonstrated in a heat map display with multivariate analyses (Fig. 6d; Fig. S5b). Altogether, these results support the concept that in severe AIM there is a unique IAV-M1 TCR repertoire that is highly cross-reactive with EBV-BM, resulting in enhanced proliferation upon acute EBV infection.

Summary analyses of cross-reactive CD8 T-cell cytokine production: severe-AIM patients have the greatest frequency of functional IFN- γ -producing cross-reactive cells *in vitro*. To determine if there were significant functional differences in cytokine production of the cross-reactive responses in the three patient groups, we examined the short-term *in vitro* cultures by using peptide pulsing in an ICS and quantified double-cytokine producers (IFN- γ ⁺ MIP-1 β ⁺) and single-cytokine producers (MIP-1 β ⁺).

Cross-reactive CD8 T cells proliferated and produced cytokines in response to cognate and cross-reactive peptide pulses with unique functional patterns in each patient group. In particular, the ratio of double-cytokine (IFN- γ ⁺ MIP-1 β ⁺) to single-cytokine (MIP-1 β ⁺) producers differed between the groups. In the severe-AIM group, both the IAV-M1 and EBV-BM short-term cultures had significantly more double-cytokine-producing (IFN- γ ⁺ MIP-1 β ⁺) cells than single-cytokine-producing (MIP-1 β ⁺) cells in response to the cognate peptide pulse than the mild-AIM and HD-SP groups (Fig. 7a). This suggests that severe-AIM patients have more EBV-BM- and IAV-M1-responding cells *in vivo* that have differentiated into IFN- γ -producing cells than mild-AIM patients or HD-SP. In contrast, in the severe- and mild-AIM groups, the EBV-BR short-term cultures had significantly fewer IFN- γ -producing cells in response to EBV-BR peptide than in the HD-SP group (Fig. 7a), further suggesting that these EBV-BR-specific cells might be partially functionally exhausted. Each donor group has a highly unique profile of cognate and cross-reactive IAV/EBV-specific IFN- γ ⁺ (Fig. 7b) and/or MIP1 β ⁺ (Fig. 7c) CD8 T cells, as demonstrated in a heat map display with multivariate analyses (Fig. S6).

Altogether, these functional studies demonstrate that each donor group had unique functional characteristics in each cognate and cross-reactive response, suggesting that

FIG 6 Legend (Continued)

costaining with pairs of tetramers (7 to 18 donors per group). In each culture, we determined the tetramer frequency of cognate (same as the culture) (a and b) and cross-reactive cells that were either single tetramer⁺ (a and b) or double tetramer⁺ (c and d). (a) Increased expansion of single tetramer⁺ cross-reactive cells in EBV-BM- and IAV-M1-stimulated cultures of cells from severe-AIM patients. There was significantly increased expansion of IAV-M1 tetramer⁺ cells in BM-stimulated cultures of cells from severe-AIM patients versus those from mild-AIM patients or HD-SP. Within the severe-AIM group, the cognate IAV-M1 tet⁺ frequency is lower than that in the mild-AIM group; instead, this group has a significantly increased frequency of cross-reactive IAV-M1 tet⁺ cells that grew in response to EBV-BM stimulation. Within the severe-AIM group, the cognate EBV-BR tet⁺ frequency is lower than that in mild-AIM patients; instead, this group has a significantly increased cross-reactive EBV-BR tet⁺ frequency in the IAV-M1-stimulated culture. Control cultures included short-term cultures with CMV pp65 or tyrosinase peptide or T2 cells (antigen-presenting cells) alone without a peptide, where the double tetramer frequencies were $\leq 0.1\%$, as indicated by the symbol φ . (b) Each donor group has unique profiles of cognate and cross-reactive IAV/EBV-specific CD8 T-cell proliferation that are highly significantly different from each other, as demonstrated in a heat map display with multivariate analyses. (c) Increased expansion of IAV/EBV double tetramer⁺ cross-reactive cells in severe (sev)-AIM patients. There were significantly higher frequencies of IAV-M1+EBV-BM tet⁺ cells in an EBV-BM-stimulated culture and IAV-M1+EBV-BR tet⁺ cells in both IAV-M1- and EBV-BR-stimulated cultures of cells from severe-AIM patients than in those of cells from mild-AIM patients or HD-SP. (The mean of all double tetramer⁺ frequencies pairing IAV-M1, EBV-BM, and EBV-BR tet⁺ with control tyrosinase tet⁺ was <0.1 ; the mean of all double tetramer⁺ frequencies in control CMV pp65-, tyrosinase-, and no-peptide-stimulated cultures was <0.1 .) (d) Severe-AIM patients have a unique profile of double tetramer⁺ cross-reactive IAV/EBV-specific CD8 T-cell proliferation, as demonstrated in a heat map display with multivariate analyses. The Student *t* test was used to compare two groups, one-way ANOVA with Sidak's multiple-comparison test was used to compare more than two groups, and multivariate analyses were done by two-way ANOVA with Tukey's multiple-comparison test with adjustment for multiple comparisons. Details of the highly significant but complex multivariate statistical analyses for the heat maps (b and d) are shown in Fig. S5.

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these responses may have been triggered differently by the same ligands *in vivo*; one way that this could occur is if the TCR repertoires for EBV and IAV epitopes differed in these donor groups. Our TCR repertoire data (Fig. 4) suggest that this is the case for the cross-reactive IAV-M1 TCR repertoire. The cross-reactive memory IAV-M1 TCR repertoire that an individual has available to be activated early immediately upon EBV infection long before the new naive EBV epitope-specific response fully arises differs between donors (43, 53) and may well determine the functional profile of these cells upon exposure to EBV antigens. Thus, the greater expansion of functional IFN- γ -producing cross-reactive responses in severe-AIM patients versus mild-AIM patients in IAV-M1- and EBV-BM-stimulated, but not EBV-BR-stimulated, short-term cultures demonstrates a strong functional cross-reactivity to EBV-BM and IAV-M1 and is consistent with the idea that this particular cross-reactivity plays a role in the mediation of AIM severity.

DISCUSSION

In these systematic studies, multiple complementary methods demonstrated that selective CD8 cross-reactive TCR repertoires (Fig. 4) between an individual's memory responses to IAV-M1 and the early antigen EBV-BM played a role in the modification of antigen-specific CD8 T-cell frequencies and functions and disease severity during the acute phase of EBV infection. Increases in the frequencies and absolute total numbers of IAV-M1, EBV-BM, IAV-M1+EBV-BM, and EBV-BR but not CMV pp65 tetramer⁺ cells were observed during AIM. However, total IAV-M1 and IAV-M1+EBV-BM cross-reactive CD8 T-cell frequencies were the only tetramer⁺ responses *ex vivo* in PBMCs that strongly directly correlated with AIM severity and that were predictive of severe disease by relative-risk analysis (Fig. 1, 2). The fact that total IAV-M1 and IAV-M1+EBV-BM cross-reactive CD8 T-cell frequencies correlated with each other suggests that the majority of the IAV-M1 tetramer⁺ cells present during AIM may be cross-reactive with EBV-BM (Fig. 2). When AIM patients were stratified into groups based on AIM severity, i.e., the mean peak frequencies in donors visiting the clinic at different times, only total IAV-M1, IAV-M1+EBV-BM and total EBV-BM tetramer⁺ CD8 T-cell counts were significantly higher in severe versus mild cases of AIM. In contrast, the mean peak frequencies of the other early EBV antigen, total EBV-BR, or cross-reactive IAV-M1+EBV-BR and EBV-BM+EBV-BR or CMV pp65 tetramer⁺ CD8 T cells did not differ between mild- and severe-AIM patients (Fig. 3). The fact that these cross-reactive memory IAV-M1 tetramer⁺ cells, which were already present at a high frequency in the donor prior to EBV infection ready to be activated immediately upon virus exposure, had unique TCR repertoires depending on whether the donor had mild or severe disease strongly suggests that these cells play a role in the mediation of disease severity. We show that there were higher numbers of IAV-M1 tetramer⁺ CD8 T cells cross-reactive with EBV-BM not only directly *ex vivo* in PBMCs (Fig. 1 to 3) but also in short-term cultures (Fig. 5 to 7). The short-term cultures further helped demonstrate that the three donor groups (severe AIM, mild AIM, and HD-SP) also had multiple qualitatively significantly different functional responses to EBV-BM and EBV-BR, as well as to cross-reactive IAV-M1 ligand. For instance, in severe-AIM versus mild-AIM patients, there was a greater proliferation of functional IFN- γ -producing (31, 33, 63) cross-reactive cells in IAV-M1 and EBV-BM than in EBV-BR-stimulated cultures (Fig. 5 to 7). Altogether, these results support the concept that cross-reactive CD8 memory T-cell responses with unique TCR repertoires and altered functional capacity play a role in determining disease severity during EBV infection and contribute to the induction of AIM.

By far, the strongest evidence that the cross-reactive IAV-M1 memory responses determine disease severity comes from an examination of the TCR repertoire use of this

FIG 7 Legend (Continued)

Sidak's multiple-comparison test was used to compare more than two groups, and multivariate analyses were performed by two-way ANOVA with Tukey's multiple-comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (P values are adjusted for multiple comparisons). All of the multiple important significant and nonsignificant differences in these graphs are shown in Fig. S6 to highlight differences between the patient groups.

population in mild- and severe-AIM patients. This study uniquely correlates the severity of a human viral disease, AIM, with T-cell repertoire use, in this case, the cross-reactive IAV-M1-specific TCR repertoire. The IAV-M1 memory TCR repertoire during AIM was completely different from that in HD-SP, as it is being driven to expand in response to cross-reactive ligands on EBV rather than its original ligand IAV-M1, most likely predominantly by EBV-BM. The cross-reactive IAV-M1 repertoire in AIM patients was much more polyclonal than in HD-SP, and it used $V\beta$ types that are commonly associated with EBV-BM, such as $V\beta 20$, -9, -2, and -29 (44, 60), instead of the classic $V\beta 19$ associated with IAV-M1 in HD-SP (Fig. 4) (5, 43, 64). What is most striking is that mild-AIM patients used different $V\beta$ families, such as $V\beta 2$ and -29, in their IAV-M1 response, and in fact, there was an inverse correlation between $V\beta 2$ use and AIM severity. Since each individual has a different memory TCR repertoire in response to IAV-M1 at the clonal level, a phenomenon known as private specificity, each person has a different IAV-specific memory cross-reactive TCR repertoire with distinct subsets that may respond to EBV-BM. Our results suggest that those individuals who had more IAV-M1 $V\beta 2$ clones cross-reactive to EBV-BM were more likely to have the mildest form of AIM. Those individuals who had the most IAV-M1 $V\beta 4.1$ memory clones cross-reactive to EBV-BM had the most severe form of AIM, with a direct correlation between the frequency of $V\beta 4.1$ use and AIM severity. This is certainly not consistent with a random activation of all IAV-M1 memory populations equally in both groups, such as that which might be expected if the viral load and virus-induced cytokines just drove the activation of all memory cells or even all cross-reactive memory cells. These results strongly suggest that the particular cross-reactive memory IAV-M1 TCR repertoire each individual had before being infected with EBV determined whether the patients developed mild or severe AIM. We have previously demonstrated that if responses are of lower avidity, as in this case, where the peptide sequences are fairly dissimilar, then this is more likely to activate a low expansion of a polyclonal population rather than a highly expanding narrow repertoire, which occurs in high-affinity often more protective cross-reactive responses (29, 60). For instance, we have observed that there is a unique high-avidity oligoclonal TCR repertoire in IAV-M1 memory cells cross-reactive with EBV-BM in the rare 5% of middle-aged individuals who never become infected with EBV (5). Disease etiology and diagnosis by TCR repertoire analysis are beginning to gain more attention as technology improves (47). Differences in virus-specific versus cross-reactive alphaherpesvirus-specific CD8 TCR repertoires have been described (65), and TCR repertoires are being linked to disease (47). For instance, using high-throughput sequencing of patients with MS, a disease associated with AIM, the TCR repertoire of cerebrospinal fluid was found to be distinct from that of blood and enriched in EBV-reactive CD8 T cells (66). Newly defined analytic tools, TCRdist and TCRdiv (repertoire distance and diversity measurements) (44), and the GLIPH algorithm (45) have been developed and may be very useful in identifying potential ligands that have been difficult to identify by using public features of the TCR repertoire that are common to donors with a particular disease like mycobacterial infection, tumors, or autoimmunity.

How, then, can different TCR repertoires influence disease outcome? Our findings are consistent with TCR activation to function differently by cross-reactive and cognate ligands. Previous studies (48, 49, 67) have shown that the TCR/CD3 complex is one of the most sophisticated immunologic signaling molecules and is capable of scalable signaling by recruiting at least 10 different immunoreceptor tyrosine-based activation motifs that control different signaling pathways (68, 69). The CD8 molecule has also been shown to be important for modulation of the TCR-MHC peptide interaction and subsequent signaling. CD8 has been shown to be particularly crucial in the activation of cross-reactive responses (70, 71). In severe-AIM patients, there was much stronger IFN- γ production by IAV-M1- and EBV-BM-specific cells than by cells with other specificities (Fig. 5 and 6). In contrast, the EBV-BR response appeared to be partially exhausted, as these T cells proliferated less and made less IFN- γ than those from HD-SP (Fig. 5 and 6). This is consistent with data that demonstrate that there is differential expression of PD1, an exhaustion marker, based on the specific TCR use of EBV-BR-

specific cells (72). Perhaps the equally prevalent cross-reactive IAV-M1+EBV-BR tetramer⁺ cells in severe-AIM EBV-BR cultures have lower avidity for EBV-BR than non-cross-reactive EBV-BR-specific cells do and are thus less likely to be exhausted. In mouse models of heterologous immunity, IFN- γ has been a major player in the mediation of severe immunopathology in the lung and in fat (17, 26, 33). We have previously reported that T-cell exhaustion is a mechanism that successfully prevents severe morbidity and death due to a large dose of an overly aggressive persistent virus, lymphocytic choriomeningitis virus (LCMV) clone 13 (73). Perhaps the fact that a large part of the EBV-BR response becomes exhausted explains why it may not directly correlate with the severity of immunopathology.

These studies also provide further evidence that tetramer and cytokine frequency measurements are two different methods to determine the sizes of antigen-specific responses and that, depending on the patient group and the antigen, they do not always correlate. For instance, in the IAV-M1 culture of the HD-SP group, there was a 3-fold higher frequency of IAV-M1 tetramer⁺ cells (50%) (Fig. 6a) than double-cytokine producers in response to an IAV-M1 pulse (15%) (Fig. 7a). In contrast, in the cross-reactive responses of the severe-AIM group in the EBV-BM culture, there was an 11-fold greater frequency of cells producing MIP-1 β in response to an IAV-M1 pulse (18% with the background subtracted) (Fig. 7a) than in the parallel IAV-M1 tetramer staining (1.6%) (Fig. 6a). This is consistent with more recent TCR sequencing data showing that tetramers may not always bind all of the predicted antigen-specific T cells (44, 45).

Hypothetically, the presence of cross-reactive IAV-M1 memory cells may alter the normal balance between EBV immune evasion strategies that normally would lead to delayed activation of CD8 responses resulting in silent persistent infection of B cells. High frequencies of cross-reactive IAV-M1 resident memory CD8 T cells in the tonsils of AIM patients may become activated early in EBV infection and may be the only CD8 T-cell response available for 3 to 4 weeks, before new naive EBV-specific CD8 T cells are activated. Unfortunately, thus far, it has not been possible to study this 4-week window between EBV exposure and the development of AIM. Perhaps their presence prevents EBV from immunosuppressing the tonsillar environment, where the virus normally moves silently from the tonsillar epithelium into B cells. This is thought to occur normally in the majority of individuals infected asymptomatically with EBV. EBV induces host IL-10, encodes a viral IL-10 homologue, and is a weak inducer of type 1 IFN (3, 12, 74). The coincident presence of activated cross-reactive IAV-M1 memory cells and EBV-induced immunosuppression may lead to dysregulated immune responses by way of early IFN- γ responses that occur during that 4-week window after EBV exposure and before the development of new naive EBV-specific CD8 T-cell responses and AIM, which leads to their overactivation.

Most likely there are other factors that may contribute to AIM induction and severity. For instance, in mild-AIM patients, there could be cross-reactive TCR repertoires that are beneficial, as suggested by the inverse correlation of some V β types with disease severity (Fig. 4). There are some hints in these studies that perhaps IAV-M1 and EBV-BR cross-reactivity may be beneficial to some patients and actually help keep disease severity milder. Also, there is a weak correlation with the viral load and AIM severity and a significantly increased relative risk if the viral load is extremely heavy ($>4.5 \log_{10}$). A heavier viral load for a protracted period of time could contribute to driving these altered immune responses in all AIM patients. However, unlike the cross-reactive IAV-M1 tetramer⁺ frequency, this factor is not consistent in all mild-AIM patients, as some mild-AIM patients have extremely heavy viral loads.

Unfortunately, studies of humans are not amenable to direct cause-and-effect testing of our hypothesis that cross-reactive CD8 memory T cells can mediate disease severity during viral infections. However, we have directly tested these theories in our mouse models multiple times by using many techniques, including cross-reactive memory CD8 T-cell adoptive transfer (49, 51). Thus far, in mouse studies, we have never found the viral load to correlate directly with the severity of pathology (50). In fact, in LCMV-immune mice infected with vaccinia virus, the viral load is almost always lighter

than that in naive controls (17, 31, 49–51). However, depending on the private specificity of the cross-reactive memory CD8 TCR repertoire, some mice develop severe fatty necrosis of their abdominal fat. Adoptive-transfer experiments showed that all three recipients of one immune donor would have the same outcome whether it was no pathology or different levels of fatty necrosis. CD8 depletion studies and anti-IFN- γ antibody treatment demonstrated that both of these factors were important in the mediation of the severity of this pathology. Our present findings are highly similar to those of our studies with IAV-immune mice challenged intranasally with LCMV, where the mice develop very severe lung pathology similar to that seen in the 1918 influenza pandemic (33). In that model, there is an increased viral load and low-affinity, low-frequency memory CD8 T cells cross-reactive to LCMV and IAV that directly mediate the severe lung pathology via excess IFN- γ . These cross-reactive responses were at such low frequency that the causal relationship needed to be shown by correlating their preinfection frequency with the subsequent severity of lung pathology, by blocking by the peptide tolerization technique, or by preventing pathology by immunizing mice with IAV mutants lacking the cross-reactive epitopes (33). Pathology could also be prevented by using anti-IFN- γ antibody. These studies suggest that early activation of low-affinity cross-reactive cells that do not expand dramatically, such as was seen here in AIM, can completely alter the immune response to the new pathogen, leading to immunopathology. In contrast, generally dominant high-affinity cross-reactive responses are more likely to lead to more oligoclonal TCR repertoires that are more protective, as we have seen in middle-aged EBV-seronegative donors (5, 24, 29).

These results suggest that an individual's history of infection, in particular, the individual's memory TCR repertoire, may help to explain variations in human disease thought previously to be only due to genetic differences, the physiological condition of the patient, or the inoculation route and dose used (17, 22, 26, 31). Additional studies are needed to clarify instances and mechanisms by which heterologous immunity may be useful or detrimental when designing vaccines and to identify individuals potentially at risk for infection-related pathology. However, it is possible that a recent episode of acute IAV infection that has activated potentially cross-reactive IAV-M1-specific resident memory T cells in the tonsils may enhance the risk of developing EBV-induced AIM. It may be that something as simple as the annual conventional killed IAV vaccination, which generates neutralizing antibodies but does not normally activate IAV-specific CD8 T cells, might help prevent AIM upon EBV exposure by decreasing acute infection with IAV and thus decreasing the likelihood that IAV-specific resident memory cells accumulate in the tonsil.

MATERIALS AND METHODS

Subjects. College students 18 to 30 years old who presented with symptoms of AIM participated in this study. Primary EBV infection was confirmed by a monospot test and the detection of EBV capsid-specific IgM in patient serum. Positive staining with HLA-A2.01+ tetramers loaded with influenza virus M1 peptide was used as an indication that these individuals had been exposed to IAV in the past (48). Patients provided up to eight blood samples (20 ml each) at entry (visit 1), 1 week (visit 2), 2 weeks (visit 3), 3 weeks (visit 4), 4 weeks (visit 5), 6 weeks (visit 6), 6 months (visit 7), and 1 year (visit 8). HLA typing was performed by screening with HLA-A2-specific MAb (BB7.2; EBioscience) staining and FACS analysis. Healthy, EBV-seropositive adult donors (HD-SP) >18 years old were used as controls. EBV infection was confirmed through the detection of EBV capsid antigen-specific (VCA) IgG antibodies in donor serum. Prior CMV infection was confirmed by CMV serology. Individual informed consent was obtained from each subject; the University of Massachusetts Medical School Human Subject Committee approved this study. The characteristics of our study subjects are summarized in Table S1.

Grouping of AIM patients by severity of disease. We developed a strategy to divide AIM patients into two groups, mild and severe AIM. Clinical measurements of disease severity collected in this study included patient-reported symptoms (malaise/fatigue, sore throat, headache, loss of appetite, myalgia, nausea, sweats, chills), physical findings on examination (adenopathy, pharyngitis, periorbital edema, hepatomegaly, and splenomegaly), with each symptom or sign rated on a scale of 1 to 10. Laboratory measurements of disease severity included the percentage of atypical lymphocytes and the CD4/CD8 ratio. A quantitative disease severity rating system that combined the severity of the most frequently observed physical finding (lymphadenopathy) with laboratory measurements of disease was calculated by using the sum of patient scores for peak percent adenopathy (clinical score by nurse: 0 to 10 \times 10), the percentage of atypical lymphocytes on a peripheral smear, and the percent CD4/CD8 ratio in PBMCs determined with the formula $\{[2 - (CD4\%/CD8\%)]/2\} \times 100$. Severe-AIM patients had total scores of ≥ 140 , and mild-AIM patients had total scores of <140 .

The combined clinical and laboratory disease severity score calculated in this manner is positively associated with the clinical disease severity score calculated by using all of the signs and symptoms in the cohort included in this study ($n = 18$, Spearman $\rho = 0.54$, $P = 0.02$), as well as in the larger AIM cohort ($n = 88$, Spearman $\rho = 0.51$, $P < 0.001$; data not shown).

PBMC isolation and short-term T-cell culture. Fresh (not frozen) PBMCs were isolated with Ficoll-Hypaque plus (Amersham Bioscience, Uppsala, Sweden) and stained with anti-CD8 antibody-coated microbeads (Miltenyi Biotech, Auburn, CA) in accordance with the manufacturer's recommendations. Positive selection of CD8 lymphocytes was performed with the Miltenyi Biotech MACS system. Short-term (3-week) CD8 T-cell cultures were set up as previously described (48). Briefly, sorted CD8 lymphocytes were plated at a 5:1 ratio with $1 \mu\text{M}$ peptide-pulsed (for >1 h), irradiated T2 cells (CRL-1992; ATCC, Manassas, VA) that were washed free of excess peptide before being mixed with CD8 T cells. CD8 T-cell lines were fed with AIM V medium (Gibco) supplemented with 14% human AB serum (Nabi, Miami, FL, or Gemini, Woodland, CA), 16% MLA-144 supernatant (75), 10 U/ml recombinant IL-2 (BD), 1% L-glutamine (Gibco), 0.5% β -mercaptoethanol (Sigma, St. Louis, MO), 1% HEPES (HyClone, Logan, UT) every 3 to 4 days and restimulated with fresh peptide-pulsed T2 cells weekly for only 3 weeks. This culture method has been optimized so as not to skew the TCR repertoire from that present *in vivo* (43, 58, 60).

HLA-A2.01-restricted peptides and MHC class I tetramers/pentamers. The following peptides at $>90\%$ purity were purchased from 21st century (Waltham, MA): EBV-BMLF1_{280–288} (GLCTLVAML), EBV-BRLF1_{109–117} (YVLDHLIVV), EBV-EBNA-3A_{509 to 604} (SVRDRLARL), EBV-LMP-2_{426–434} (CLGGLLTMMV), EBV-LMP-2_{329–337} (LLWTLVLL), CMV pp65_{495–503} (NLVPMVATV), IAV-M1_{58–66} (GILGFVFTL), IBV-NP_{85–94} (KLGEFYNQMM), and human endogenous tyrosinase_{369–377} (YMNGTMSQV). Tetramers were assembled with these peptide sequences for EBV-BMLF1, EBV-BRLF1, IAV-M1, CMV pp65, and tyrosinase by the tetramer facility at University of Massachusetts Medical School as previously described (48, 72). MHC class I pentamers for IAV-M1 and EBV-BMLF1 were purchased from ProImmune (Oxford, United Kingdom).

Extracellular and intracellular staining. Sorted CD8 T cells isolated from fresh PBMCs directly *ex vivo* or short-term-cultured CD8 T cells were plated at 10^6 /well in U-bottom 96-well plates (Sigma-Aldrich) and washed with staining buffer (phosphate-buffered saline, 2% fetal calf serum, 1% sodium azide). The first tetramer was incubated at room temperature for 20 min, and the excess was washed off before the addition of a second tetramer. In experiments where cells were costained with tetramers and/or pentamers, and with additional MAbs for surface markers (BD), such as CD3 (clone UCHT1), CD4 (clone RPA-T4), and CD8 (clone SK1), the tetramers were washed off prior to addition, and these MAbs were incubated for 20 min at room temperature as previously described (48). Cells stained in accordance with the manufacturer's protocols were either fixed with FACS lysing solution or permeabilized with Cytotfix/Cytoperm for intracellular assays. Anti-IFN- γ (clone B27) and anti-MIP-1 β (clone D-21-1351) MAbs were used for ICS. All antibodies and reagents were purchased from BD. Flow cytometry was done with LSRII (Beckman Coulter, Inc., Fullerton, CA).

EBV quantification. Genomic DNA was extracted from enriched B cells with the DNeasy kit (Qiagen, Valencia, CA) (72, 76). Each sample was normalized to contain the DNA of a total of 10^6 B cells. Samples were run in duplicate by using the recommended protocol accompanying a LightCycler EBV quantification kit (Roche Diagnostics, Indianapolis, IN). An internal positive control was supplied with this kit.

TCR V β analysis. Sorted CD8 T cells isolated from fresh PBMCs directly *ex vivo* were incubated for 20 min with IAV-M1-specific tetramer, which was then washed off. An additional 20-min incubation was performed with 24 TCR V β antibodies that cover $>70\%$ of the commonly used human V β types (IOTest Beta Mark TCR V β Repertoire kit; Beckman Coulter, Inc., Fullerton, CA). Samples were read on an LSRII (Beckman Coulter, Inc., Fullerton, CA). IMGT TCR gene nomenclature was used to define TCR V β types.

TCR-tetramer blocking determination. Sorted CD8 T cells isolated from fresh PBMCs directly *ex vivo* or short-term-cultured CD8 T cells from AIM patients were incubated with IAV-M1-, EBV-BM-, and EBV-BR-specific tetramers as described for extracellular staining. Since blocking of tetramer binding by the cross-reactive tetramer was a common event, we always used the following protocol for tetramer staining in order to accurately evaluate the frequency of cross-reactive CD8 T cells. Our panel for tetramer staining of CD8 T cells always included (i) each epitope-specific tetramer alone (IAV-M1, EBV-BM, EBV-BR, tyrosinase), (ii) generally non-cross-reactive control self-peptide specific tyrosinase tetramer with the epitope-specific tetramers together (IAV-M1, EBV-BM, EBV-BR) and (iii) a combination of double tetramer costaining with the following pairs: IAV-M1 plus EBV-BM, IAV-M1 plus EBV-BR and EBV-BM plus EBV-BR. Stained samples were read on an LSRII (Beckman Coulter, Inc., Fullerton, CA), and the percentage of tetramer⁺ cells and mean fluorescent intensity (MFI) of tetramer⁺ cells were analyzed with the FlowJo version 9.9.3 program. The estimated tetramer-blocking level for each tetramer was calculated by the formula (costained tetramer A MFI/alone tetramer A MFI) \times (costained tetramer A%/alone tetramer A%) \times 100 (Fig. 1; Fig. S2a and b).

Statistical analyses. Statistical analyses were performed with Prism version 6 (GraphPad Software, Inc.). The tests used included the Student *t* test, the linear regression test, and the nonparametric Mann-Whitney U test. The SDI was calculated as follows: $D = \sum n(n-1)/N(N-1)$, where D is diversity, n is the number of individual clonotypes, and N is the number of unique clonotypes (58). The Student *t* test was used to compare two groups, and one-way ANOVA with Sidak's or Tukey's multiple-comparison test was used to compare more than two groups. To display pairwise correlations between variables of interest, we computed a correlation matrix by using the Pearson correlation coefficient. We then graphically displayed the matrix with the corplot R package (<https://cran.r-project.org/web/packages/corplot/>), which represents correlation values as circles with areas and color shades proportional to correlation values.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.01841-17>.

FIG S1, TIF file, 44.7 MB.

FIG S2a, TIF file, 44.7 MB.

FIG S2b, TIF file, 44.7 MB.

FIG S3, TIF file, 44.7 MB.

FIG S4, TIF file, 25.1 MB.

FIG S5, TIF file, 44.7 MB.

FIG S6, TIF file, 44.7 MB.

TABLE S1, DOCX file, 0.1 MB.

TABLE S2, DOCX file, 0.1 MB.

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